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Molecular Cloning and Functional Expression of KCNQ5, a Potassium Channel Subunit That May Contribute to Neuronal M-current Diversity*

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We have isolated KCNQ5, a novel human member of the KCNQ potassium channel gene family that is differentially expressed in subregions of the brain and in skeletal muscle. When expressed in *Xenopus* oocytes, KCNQ5 generated voltage-dependent, slowly activating K^+-selective currents that displayed a marked inward rectification at positive membrane voltages. KCNQ5 currents were insensitive to the K^+ channel blocker tetraethylammonium but were strongly inhibited by the selective M-current blocker linopirdine. Upon coexpression with the structurally related KCNQ3 channel subunit, current amplitudes increased 4–5-fold. Compared with homomeric KCNQ5 currents, KCNQ3/KCNQ5 currents also displayed slower activation kinetics and less inward rectification, indicating that KCNQ5 combined with KCNQ3 to form functional heteromeric channel proteins. This functional interaction between KCNQ5 and KCNQ3, a component of the M-channel, suggests that KCNQ5 may contribute to a diversity of heteromeric channels underlying native neuronal M-currents.

Voltage-dependent potassium channels are key regulators of the resting membrane potential and modulate the excitability of electrically active cells, such as neurons and myocytes. Several classes of voltage-dependent K^+ channels have been cloned, and probably all form oligomeric proteins through the assembly of four α-protein subunits. The tetrameric pore complex can further interact with auxiliary subunits to enhance and/or modify currents mediated by the pore-forming α-subunits.

The KCNQ family of voltage-dependent K^+ channels was originally established by positional cloning of the *KCNQ1* gene (1), which encodes a K^+ channel protein (KvLQT1) with six transmembrane domains and a characteristic pore region. So far, the KCNQ family consists of four members, all of which are associated with hereditary human diseases. KCNQ1 functionally interacts with KCNE1 (IsK) (2), a small β-subunit protein with a single transmembrane domain, to generate the slowly activating delayed rectifier I_{Ks} current of cardiomyocytes (3–6). Deleterious mutations in either subunit result in prolongation of the cardiac action potential and an increased risk of ventricular arrhythmia in patients with long QT-syndrome (7–10). Both KCNQ1 and KCNE1 are also expressed in the inner ear, and a class of recessive mutations of either gene is associated with hearing loss (11, 12). In intestine, KCNQ1 probably associates with the IsK-like KCNE3 protein (13) to generate a distinct K^+ current (14). This KCNQ1/KCNE3 channel complex may represent the native basolateral cAMP-regulated K^+ conductance in colonic crypt cells (15), important for apical cAMP-stimulated chloride secretion associated with secretory diarrhea and cystic fibrosis.

KCNQ2 and KCNQ3 express and colocalize in various subregions of the brain (16–20). KCNQ2 and KCNQ3 were cloned by linkage to benign familial neonatal convulsions, a form of epilepsy in human infants. Missense mutations in either KCNQ2 or KCNQ3 are associated with benign familial neonatal convulsions (16, 21, 22). Since epilepsy is due to an electrical hyperexcitability in the brain, members of the *KCNQ* gene family may play an important stabilizing role in the nervous system. Whereas KCNQ2 expresses K^+ currents very similar to KCNQ1 (16, 17, 19, 20), KCNQ3 alone produces much smaller amplitude currents (17, 19). Coexpression in *Xenopus* oocytes of both KCNQ2 and KCNQ3 results in currents that are about 10-fold larger than those expressed by KCNQ2 alone (17–19) and that also display altered biophysical and pharmacological properties, suggesting that novel channels are formed by the heteromeric association of KCNQ2 and KCNQ3 subunits. The biophysical and pharmacological profiles of KCNQ2/KCNQ3 currents expressed in *Xenopus* oocytes are very similar to that of the native neuronal M-type K^+ current (19, 23), which is thought to be a prominent regulator of neuronal excitability. Similar to the native M-current, KCNQ2/KCNQ3 channel activity is strongly reduced by muscarinic acetylcholine agonists (19, 24, 25), and therefore it is now accepted that KCNQ2/KCNQ3 heteromeric channels form the native M-channel.

KCNQ4, another member of this gene family, is expressed in sensory outer hair cells of the cochlea and is mutated in one form of nonsyndromic autosomal dominant deafness (DFNA2) (26, 27). Interestingly, coexpression of KCNQ3 with KCNQ4 in *Xenopus* oocytes also increases current amplitudes (26), although to a far less extent than observed with KCNQ2/KCNQ3 coexpression. This raises the possibility that different KCNQ channel subunits can combine to produce variants of M-currents in different parts of the nervous system.

In the present work, we have cloned and characterized a novel member of the KCNQ extended gene family. When functionally expressed in *Xenopus* oocytes KCNQ5 produced K^+

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channels activated by depolarization, with kinetic properties similar to other KCNQ channels. KCNQ5 was expressed in skeletal muscle and in the brain, where its expression pattern overlaps with those of KCNQ2 and KCNQ3, which underlie the native M-current. Similar to KCNQ2, KCNQ5 formed functional heteromers with KCNQ3 that produced larger current amplitudes and were sensitive to block by linopirdine, an M-channel-specific inhibitor, suggesting that neuronal M-channels may possibly include heteromeric variants composed of KCNQ3, combined with KCNQ2, KCNQ5, or other members of the *KCNQ* gene family.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Expression of KCNQ5—The *KCNQ5* gene was initially identified as a genomic survey sequence (bacterial artificial chromosome clone) in a homology search of GenBank™ (accession number AQ344243). Using this sequence, a human brain cDNA library (Edge BioSystems) was screened. A composite full-length cDNA construct was assembled from two overlapping cDNA clones and subcloned into the *Xenopus* expression vector pSGEM (28). The cDNA was fully sequenced on both strands using an automated DNA sequencer (ABI 310). For *Xenopus* oocyte expression, capped cRNA was synthesized using the T7 mMessage mMachine kit (Ambion). For Northern blot analysis, a DIG[1]-labeled riboprobe of 1.6 kb in length (containing mainly C-terminal sequences) was generated with the DIG RNA Labeling kit (Roche Molecular Biochemicals) according to the manufacturer's instructions and hybridized to a series of human RNA blots (CLONTECH). Membranes were exposed for 4 min on a Lumi-Imager (Roche Molecular Biochemicals).

Chromosomal Localization of KCNQ5—Fluorescence *in situ* hybridization (Genome Systems) analysis was performed to determine the chromosomal localization of KCNQ5. Briefly, purified DNA from the bacterial artificial chromosome clone (AQ344243) was labeled with digoxigenin dUTP by nick translation, and the labeled probe was hybridized to human metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. KCNQ5-specific signals were detected on the long arm of chromosome 6 using fluoresceinated antidigoxigenin antibodies followed by counterstaining with 4′,6-diamidino-2-phenylindole.

Electrophysiology—*Xenopus laevis* oocytes were obtained from tricaine-anesthetized animals. Ovaries were collagenase-treated (1 mg/ml; Worthington, type II) in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) for 120 min and subsequently stored in recording solution ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) with additional sodium pyruvate (275 mg/liter), theophylline (90 mg/liter), and gentamycin (50 mg/liter) at 18 °C. Oocytes were individually injected with 10 ng of cRNA encoding hKCNQ5, rKCNQ3, hKCNQ2, and hKCNQ1 or coinjected with 10 ng of hKCNQ5 plus 5 ng of hIsk (KCNE1), hMiRP1 (KCNE2), hMiRP2 (KCNE3), or mMiRP3 (KCNE4), respectively. rKCNQ3 was cloned from a λZAP cDNA library (Stratagene) using a DIG-labeled (Roche Molecular Biochemicals) expressed sequence tag clone (GenBank™ accession number AA019129) as a probe. hMiRP1 and hMiRP2 were cloned from human genomic DNA, and mMiRP3 was cloned from murine brain cDNA using primers derived from the published sequences (13). Standard two-electrode voltage clamp recordings were performed at room temperature with a Turbo Tec 10CD (NPI) amplifier, an ITC-16 interface combined with Pulse software (Heka) and Origin version 5.0 (Microcal Software) for data acquisition on a Pentium II PC. Macroscopic currents were recorded 2–4 days after injection. The pipette solution contained 3 M KCl. All fitting procedures were based on the simplex algorithm. Student's *t* test was used to test for statistical significance, which was assumed if $p < 0.05$ and indicated by an asterisk. GenBank™ accession numbers for sequences used are as follows: hKCNQ1, AJ006345; hKCNQ2, NM004518; rKCNQ3, AF087454; KCNE1/hIsk, M26685; KCNE2/hMiRP1, AF071002; KCNE3/hMiRP2, AF076531; KCNE4/mMiRP3, AF076533.

RESULTS AND DISCUSSION

Cloning and Tissue Distribution of KCNQ5—A search of the GenBank™ data base revealed a human bacterial artificial chromosome end sequence (AQ344243) with significant homology to the KCNQ potassium channel family. The sequence information was used to isolate overlapping cDNA clones from a human brain cDNA library (Edge BioSystems), and two cDNA clones were assembled to generate a full-length cDNA clone. The initiator methionine of the cDNA was assigned to the first ATG in frame and is preceded by a stop codon in the same frame. The full-length KCNQ5 cDNA encodes a protein of 932 amino acids (Fig. 1) with a predicted molecular mass of ~102 kDa. Hydropathy analysis supported a topological model with six transmembrane domains. KCNQ5 shows significant homology to other KCNQ predicted proteins, with KCNQ4 being the closest relative (65% identity). KCNQ5 is about 50% identical to KCNQ3 and KCNQ2 but only 40% to KCNQ1. Homology is observed, in particular, throughout the membrane-spanning regions and the conserved pore region between transmembrane segments S5 and S6. Predicted KCNQ proteins are characterized by long C-terminal tails. Among the known KCNQ proteins, KCNQ5 has the longest C terminus, followed by KCNQ2 and KCNQ3. A distinct region of high sequence conservation was found in the KCNQ5 C-terminal tail, shared with the other KCNQ proteins. This region is frequently the site of clinical mutations of other KCNQ proteins associated with hereditary diseases (8, 12, 17). A recent report suggests that deletion of the conserved C-terminal domain of KCNQ1 found in a Jervell and Lange-Nielsen syndrome pedigree disrupts homotypic subunit assembly (29). In addition, in a benign familial neonatal convulsions pedigree, a 56-amino acid extension in the length of the KCNQ2 C-terminal tail caused by a frameshift mutation at the 3′-end of the open reading frame, lowers current amplitudes drastically when expressed in *Xenopus* oocytes. In contrast, truncation of the last seven natural amino acids increases channel activity 2-fold (30). Together, these results stress the functional importance of the C-terminal region in the KCNQ proteins.

The KCNQ5 protein contains numerous potential sites for phosphorylation by protein kinase C but lacks an N-terminal consensus site for cAMP-dependent phosphorylation that is present in KCNQ1 and KCNQ2. KCNQ1 and I_{Ks} currents are stimulated by protein kinase A (6, 31), whereas conflicting data have been reported concerning the effect of protein kinase A on KCNQ2 currents (17, 20).

Northern blot analyses (Fig. 2) detected a 7.5-kb KCNQ5 transcript in human adult skeletal muscle and brain. In the brain, KCNQ5 transcripts are widely distributed with strongest expression in cerebral cortex, occipital pole, frontal lobe, and temporal lobe. Lower levels of expression were detected in hippocampus and putamen. The expression pattern of KCNQ5 in the brain is very similar to that previously described for KCNQ2 and KCNQ3 (16–18), with transcript sizes of 8.5 and 10.5 kb, respectively. Notably, in contrast to KCNQ2, KCNQ5 is greatly reduced or absent in cerebellum. KCNQ5 probably is the only KCNQ channel subunit strongly expressed in skeletal muscle. However, the presence of N-terminal splice variants of KCNQ1 (6) and faint expression levels of KCNQ4 (26) has been reported in skeletal muscle as well. In contrast to the presence of transcripts, KCNQ-like currents have not been observed in skeletal muscle. Whether KCNQ5 and related KCNQ channels contribute to electrical signaling in skeletal muscle, therefore, remains unknown.

Expression of KCNQ5 in Xenopus Oocytes—The possible function of KCNQ5 as a potassium channel was addressed by electrophysiological analysis of *Xenopus* oocytes injected with KCNQ5 cRNA transcribed *in vitro*. Two to four days after injection, novel currents were detected that were not observed in water-injected control oocytes. Currents activated very

[1] The abbreviations used are: DIG, digoxigenin; I_{Ks}, slow component of the cardiac delayed rectifier current; kb, kilobase(s); TEA, tetraethylammonium.

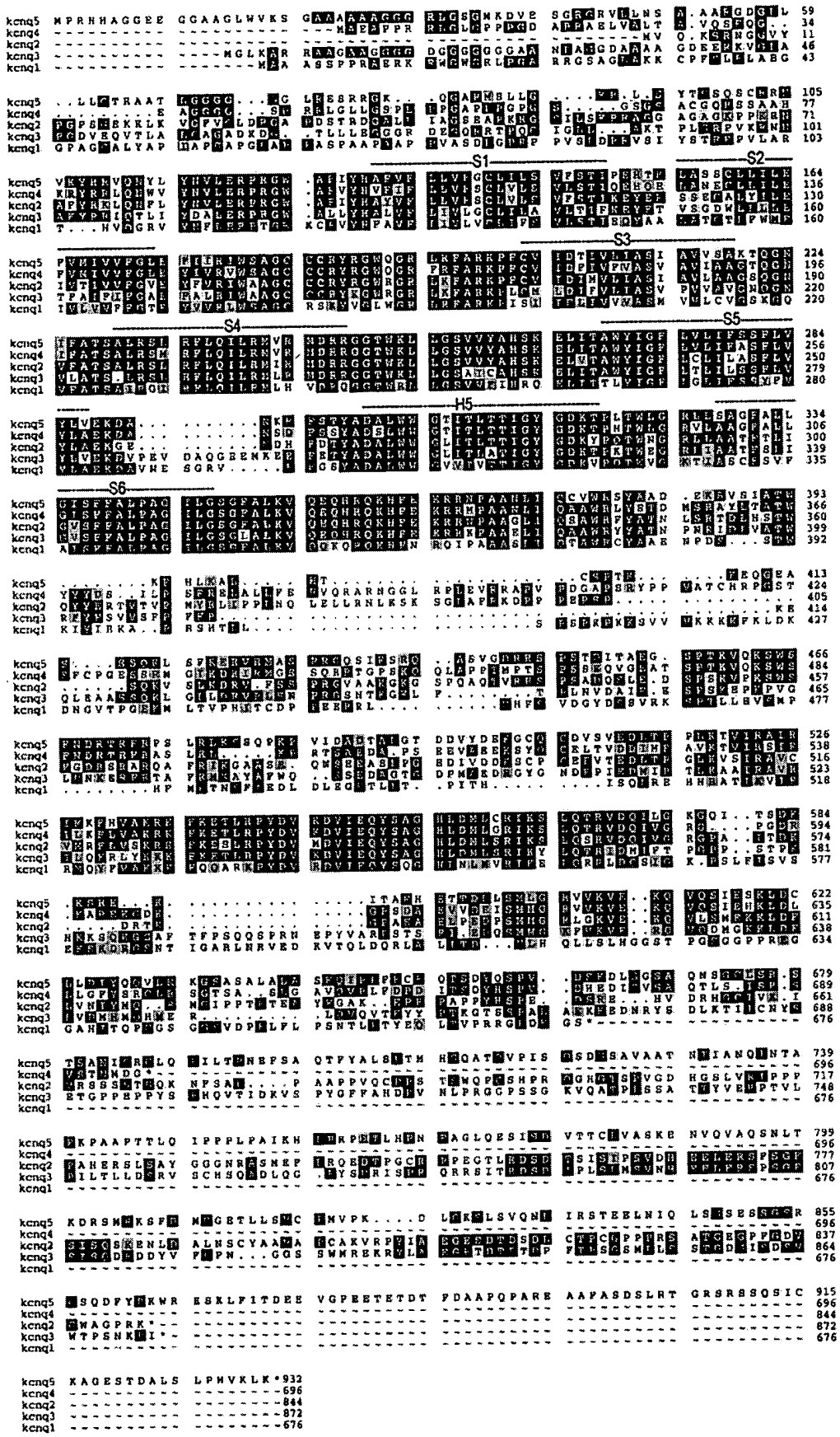

FIG. 1. **Protein sequence of KCNQ5 and comparison with other KCNQ proteins.** Alignment of human KCNQ5 with human KCNQ1, KCNQ2, KCNQ3, and KCNQ4 is shown. Identical and conserved amino acids are *boxed* in *black* and *gray*, respectively. The six putative transmembrane domains S1 through S6 and the pore region H5 are indicated by the *stippled lines*. The KCNQ5 sequence has been deposited in the GenBank™ data base under the accession number AF249278.

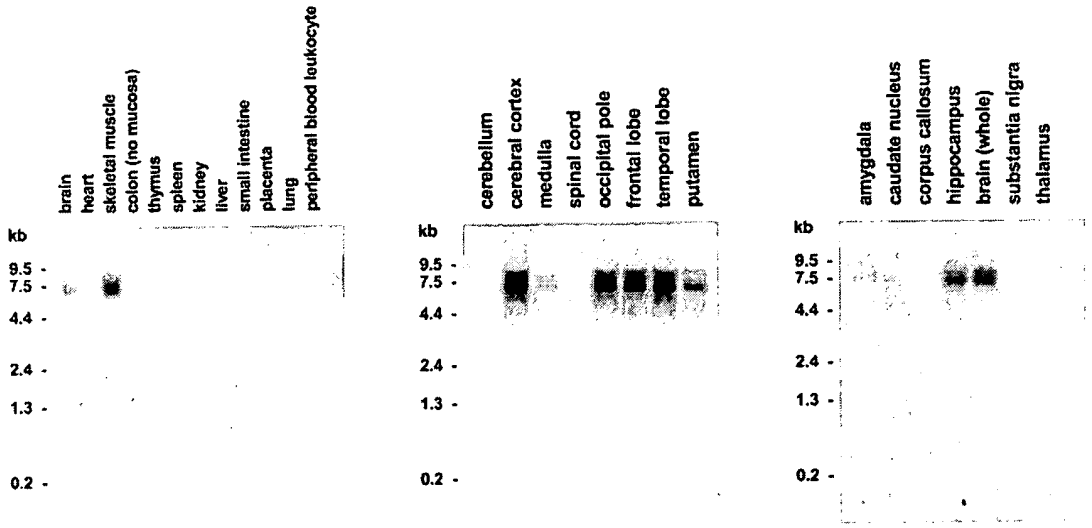

FIG. 2. **Tissue distribution of human potassium channel KCNQ5.** Northern blots of multiple human tissues and subregions of human brain containing poly(A)$^+$ RNA (CLONTECH), hybridized with a KCNQ5-specific DIG-labeled RNA probe.

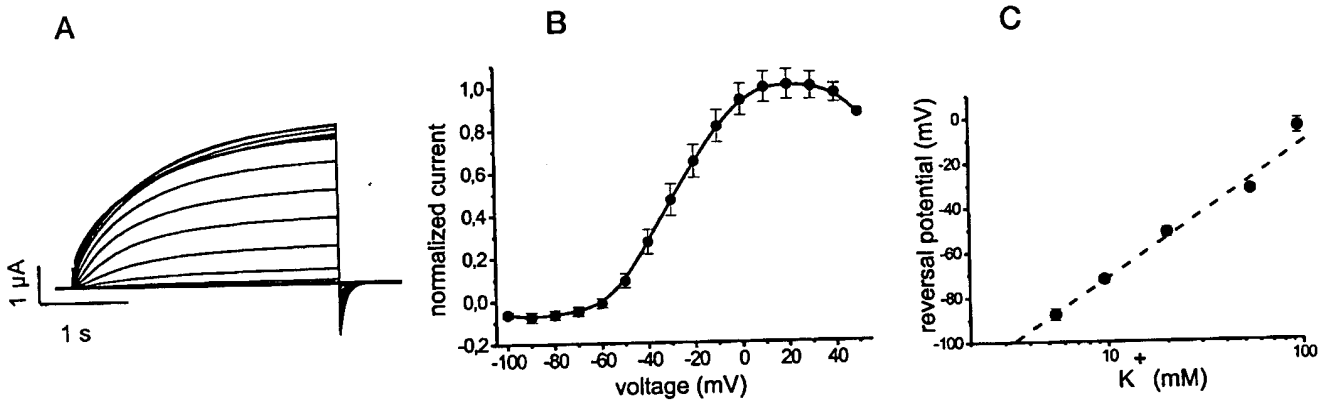

FIG. 3. **Functional expression of KCNQ5 in *Xenopus* oocytes.** *A*, representative current traces from a KCNQ5-injected oocyte subjected to a pulse protocol stepping the membrane potential from holding potential (−100 mV) to test pulses from −100 to +50 mV, and then back to holding potential. *B*, *I*-V relationships from KCNQ5 currents recorded in oocytes (n = 12), using the protocol in *A*. Current amplitudes were normalized to maximum value. *C*, tail current reversal potentials of KCNQ5 currents as a function of extracellular K^+ concentration (n = 5). The *dashed line* is calculated by the Nernst equation corresponding to a perfectly selective K^+ channel.

slowly (Fig. 3*A*) and were not fully activated even after 3-s test pulses. At lower step potentials, KCNQ5 currents showed a delay in activation, similar to KCNQ1 currents (32). In most cells (in 23 out of 28 oocytes) activation traces above +20 mV displayed a "crossover" phenomenon, which was observed independently of current amplitudes. Activation of KCNQ5 currents was generally slower than that of other KCNQ currents and could be well described by a second order exponential function, with time constants of 119 ± 7 and 929 ± 51 ms at +40 mV (Table I). Current-voltage relationships of KCNQ5 currents are shown in Fig. 3*B*. Currents were activated at depolarizing potentials positive to −60 mV and displayed marked inward rectification at potentials greater than 0 mV (Fig. 3, *A* and *B*). Strong inward rectification only has been demonstrated for the related KCNQ3 channel (18). KCNQ5 deactivation was fitted to a second order exponential function. Two components of deactivation, with time constants of 51 ± 2 and 281 ± 24 ms, were observed at a repolarizing voltage of −100 mV, following a 3-s depolarizing step to +40 mV (Table I). KCNQ1 tail currents display a characteristic "hook" indicative of recovery from inactivation (32, 33). We did not observe such a feature for KCNQ5 currents at voltages between −100 and +40 mV. Additionally, double-pulse protocols commonly used to reveal inactivation (33) also failed to reveal inactivation (not shown).

To examine the K^+ selectivity of KCNQ5, tail current reversal potentials were determined in bath solutions containing 5.4, 9.6, 20, 54, and 96 mM K^+. A 10-fold reduction in external K^+ shifted the reversal potential by about 58 mV (Fig. 3*C*), indicating nearly perfect selectivity for K^+.

Next we investigated the pharmacological properties of KCNQ5 (protocols as described in the legend to Fig. 4*D*; data not shown). KCNQ5 currents were only weakly sensitive to the nonselective K^+ channel blocker TEA. The IC_{50} value of TEA was ≫30 mM and similar to results obtained with KCNQ3 (18, 19, 34). Consistently, both channel proteins contain threonine residues within the pore region at a position that mainly determines sensitivity to external blockade by TEA. While in KCNQ2 a tyrosine residue at this position confers high TEA sensitivity (19, 35), other residues may be responsible for the intermediate TEA sensitivity of KCNQ1 and KCNQ4 (6, 34). Another nonspecific potassium channel blocker, quinidine, at 300 µM blocked KCNQ5 currents by 50%. Specific inhibitors of KCNQ1 and I_{Ks} were tested at concentrations that nearly completely block these currents. The chromanol 293B (36) at 100 µM blocked KCNQ5 currents by 45%. In comparison, KCNQ1 is

TABLE I

Activation and deactivation time constants of homomeric and heteromeric KCNQ channels

Oocytes were injected with 10 ng of either KCNQ5 or KCNQ2 cRNA or with a mixture of 10 ng each of KCNQ5 and KCNQ3 or of KCNQ2 and KCNQ3 cRNA. Currents were measured and fitted for activation and deactivation time constants as described in the text and under "Experimental Procedures," respectively. Asterisks indicate significance of homomeric *versus* heteromeric expression. Values are mean ± S.E.; numbers of oocytes are shown in parenthesis.

Constants	KCNQ5	KCNQ5/KCNQ3	KCNQ2	KCNQ2/KCNQ3
		ms		
Activation, τ_{fast}	119 ± 7 (22)	167 ± 14* (14)	40 ± 6 (10)	73 ± 6* (10)
Activation, τ_{slow}	929 ± 51 (22)	857 ± 32 (13)	291 ± 71 (10)	490 ± 31* (9)
Deactivation, τ_{fast}	51 ± 2 (10)	45 ± 2 (13)	29 ± 5 (9)	47 ± 3* (6)
Deactivation, τ_{slow}	281 ± 24 (14)	349 ± 81 (13)	241 ± 64 (8)	226 ± 29 (6)

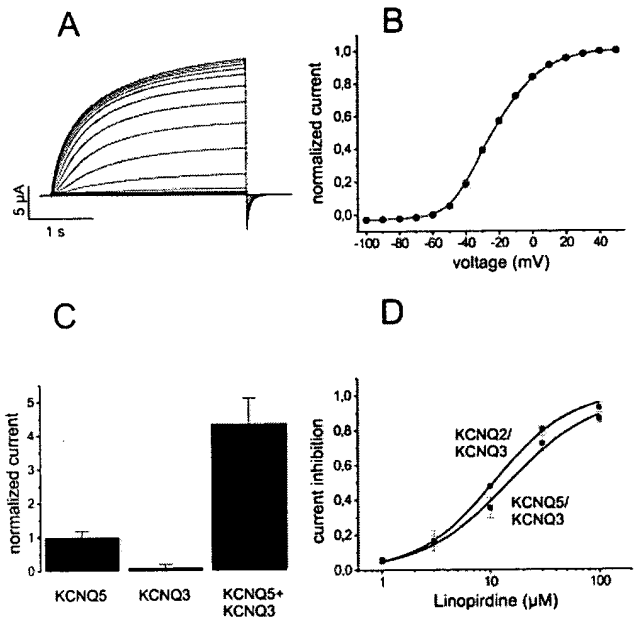

FIG. 4. **Functional interaction of KCNQ5 with KCNQ3.** *A*, representative current traces from an oocyte coinjected with KCNQ5 and KCNQ3 using the same voltage protocol as in Fig. 3*A*. *B*, *I*-V relationships of oocytes expressing KCNQ5 plus KCNQ3 (n = 11). Current amplitudes normalized to maximum value. *C*, bar graphs plotting mean current amplitudes at the end of a 3-s test pulse to +40 mV, from a holding potential of −100 mV. Oocytes injected with cRNA encoding KCNQ5 (n = 14), KCNQ3 (n = 6), or KCNQ5 plus KCNQ3 (n = 12), the amount of RNA injected as described in Table I. Current amplitudes normalized to the amplitude of KCNQ5. *Error bars* indicate S.E. *D*, concentration dependence of inhibition of KCNQ2/KCNQ3 and KCNQ3/KCNQ5 by linopirdine. Holding potential was −100 mV, 3-s test pulse, to 0 mV every 10 s. Each concentration was perfused for 5 min. Inhibition values were obtained at the end of the depolarizing step and normalized to control (averaged from five oocytes). *Error bars* indicate S.E.

80% blocked, and I_{Ks} is completely blocked at this concentration (37). The class III antiarrhythmic agent clofilium blocks KCNQ1 with an IC_{50} of <10 μM (6). At 30 μM, clofilium reduced KCNQ5 current by 40%, similar to its inhibitory effect on KCNQ3 (30% inhibition at 10 μM). Clofilium produces little inhibition of KCNQ2 at 10 μM (18). Thus, the general pharmacological properties of KCNQ5 are distinct from KCNQ1 and more similar to those of KCNQ3 than to KCNQ2. Most importantly, linopirdine, a specific blocker of the neuronal M-channel very effectively inhibited KCNQ5 with a K_d value of 16 ± 1 μM.

Functional Interaction of KCNQ5 and KCNQ3—All members of the KCNQ family form functional heteromeric complexes with homologous or structurally different K^+ channel subunits. To investigate the possibility that KCNQ5 might also form heteromeric channels, we coexpressed KCNQ5 with other KCNQ proteins and with cDNAs encoding different KCNE proteins.

After coexpression with either KCNQ1 or KCNQ2, or with any of the four known KCNE proteins (KCNE1 through KCNE4), we failed to detect evidence for functional interaction of any of these proteins with KCNQ5. However, coexpression of KCNQ5 with KCNQ3 (Fig. 4*A*) produced currents strikingly different from those produced by either KNCQ5 or KCNQ3 alone. Whereas KCNQ3 expressed very small amplitude currents almost indistinguishable from background level, in agreement with previous findings (17, 19), coinjection with KCNQ5 yielded 4–5 times larger currents than KCNQ5 alone (Fig. 4, *A* and *C*). There was no significant shift in the voltage dependence of the currents (Fig. 4*B*), but the *I*-V relationship indicated that KCNQ5/KCNQ3 currents were less inwardly rectifying at positive membrane potentials.

The increase in current amplitude was accompanied by slight changes in activation kinetics, as shown in Table I. Coexpression of KCNQ5 with KCNQ3 significantly slowed the fast component of activation (KCNQ5/KCNQ3 τ_{fast} = 167 ± 14 ms; KCNQ5 τ_{fast} = 119 ± 7 ms) without altering the slow activation component and the deactivation kinetics (see Table I). Compared with KCNQ2/KCNQ3, heteromeric KCNQ5/KCNQ3 channels exhibited slower activation, whereas deactivation kinetics of both heteromers were very similar (see Table I).

The large increase in current amplitudes, the differences in gating kinetics observed after coexpression, and the colocalization of KCNQ5 with KCNQ3 in distinct subregions of the brain all indicate that KCNQ5 can associate with KCNQ3 to form functional heteromeric channels.

The functional interaction between KCNQ5 and KCNQ3, a molecular component of the neuronal M-current, and the sensitivity of KCNQ5 to the M-channel blocker linopirdine suggest that KCNQ5 may also contribute to the molecular diversity of this physiologically important current.

For better comparison of the kinetic properties of KCNQ3/KCNQ5 heteromers with those of the native M-current and with KCNQ2/KCNQ3 currents heterologously expressed in *Xenopus* oocytes, we used a classical M-current protocol. Starting from a holding potential of −30 mV, we stepped to −50 mV and determined deactivation kinetics as described by Wang *et al.* (19). The data obtained for KCNQ3/KCNQ5 (τ_{fast} = 199 ± 10 ms; τ_{slow} = 985 ± 60 ms; n = 6) and KCNQ2/KCNQ3 (τ_{fast} = 209 ± 11 ms; τ_{slow} = 766 ± 37 ms; n = 6), respectively, correspond well to the time constants reported for the native M-current (τ_{fast} = 103 ms; τ_{slow} = 1041 ms) (19). In addition, similar to homomeric KCNQ5 currents, also heteromeric KCNQ3/KCNQ5 channels were strongly inhibited by linopirdine. As shown in Fig. 4*D*, sensitivity to block by linopirdine of KCNQ3/KCNQ5 was very similar to that obtained for KCNQ2/KCNQ3 (IC_{50} values of 15 ± 2 μM and 10 ± 1 μM, respectively), further corroborating the M-like nature of the KCNQ3/KCNQ5 current.

M-currents are found in peripheral and central neurons but have not been described in cerebellum (19). Interestingly, KCNQ5 and KCNQ3 transcripts are only weakly or not ex-

pressed in cerebellum (17–19), in contrast to KCNQ2, which is strongly expressed (16, 18, 19).

KCNQ4 also can functionally interact with KCNQ3 (26) to yield M-like currents, raising the possibility that heterogeneous populations of M-channels exist within the peripheral and central nervous system, varying in kinetic and pharmacological properties. Heterogeneity in the molecular composition of M-like channels may possibly be further increased by contributions of K^+ channels from other potassium channel gene families (38, 39).

The possibility that the KCNQ5 protein may contribute to M-currents within the brain renders the *KCNQ5* gene a candidate genetic locus for epileptic disease. By fluorescence *in situ* hybridization analysis, we mapped the *KCNQ5* gene to chromosome 6q14. There are two known loci on chromosome 6 linked to epileptic diseases, a cloned gene at 6q24 (*EPM2A*), which is defective in progressive myoclonic epilepsy type 2 (40, 41), and an unknown gene mapped to 6p12-p11, which is responsible for juvenile myoclonic epilepsy (JME) (42). Although our mapping results probably exclude involvement of KCNQ5 in JME, the identification of KCNQ5 makes it now possible to investigate possible associations with other forms of epilepsy or inherited neurological diseases.

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